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Mutations in the Nonstructural Protein 5A Gene and Response to Interferon Therapy in Young Patients With Chronic Hepatitis C Virus 1b Infection

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A region associated with sensitivity to interferon (IFN) has been identified previously in the nonstructural protein 5A (NS5A) of hepatitis C virus (HCV) genotype 1b. A study was undertaken to determine whether the presence of mutations in the $NS5A_{2209–2248}$ sequence could serve as a predictor of response to IFN therapy in children and adolescents with chronic HCV-1b infection. Sixteen children (M/F ratio = 11:5; mean age 11.7 years, range 5 to 19 years) with chronic HCV-1b infection who received IFN-α for 6 months (total dose: 8 MU/kg) were enrolled in this study. Twelve of the children (75%) had an underlying malignant disease. Pretreatment NS5A gene sequences were detected by reverse transcription–nested polymerase chain reaction (RT-nested PCR). PCR products were subjected to direct sequencing by the dideoxy chain termination method. The amino acid sequences of $NS5A_{2209–2248}$ were compared with the published $NS5A_{2209–2248}$ sequences of HCV-J. The $NS5A_{2209–2248}$ sequences were detected in 10 (63%) of the 16 children. Eight patients had the wild-type sequences, with no amino acid changes; and two patients had the intermediate type, with only one amino acid change. Four (25%) of the 16 patients responded completely to IFN therapy. Three of the four patients had the wild-type sequences, while none of the patients with the mutant type had a complete response. Serum HCV RNA levels in children with the wild type did not differ from those in patients with the mutant type. This study shows that there is no significant correlation between response to IFN and mutations in $NS5A_{2209–2248}$. The amino acid sequences in $NS5A_{2209–2248}$ in young patients with chronic HCV-1b infection appear to be conserved. ***J. Med. Virol. 53:361–365, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: children and adolescents; polymerase chain reaction; direct sequencing; acute leukemia; blood transfusion

INTRODUCTION

It was reported previously that the efficacy of interferon (IFN) therapy in children with chronic hepatitis C is influenced by clinical and virologic factors, such as hepatitis C virus (HCV) genotype, serum HCV RNA level, histologic activity of liver biopsy specimens, and underlying disease [Fujisawa et al., 1994, 1995; Komatsu et al., 1996]. Although the response to IFN therapy is very poor in both adults and children with chronic HCV genotype 1b (HCV-1b) infection [Fujisawa et al., 1995; Martinot-Peignoux et al., 1995; Garson et al., 1995], HCV-1b predominates in Asian countries, including Japan [Takada et al., 1993; Mahaney et al., 1994; Noubaum et al., 1995]. Recently, a small region in the nonstructural protein 5A ($NS5A_{2209–2248}$) of HCV-J was reported to be associated closely with sensitivity to IFN therapy, and the number of amino acid changes in $NS5A_{2209–2248}$ before IFN therapy is considered an accurate predictor of response to IFN therapy in adults with chronic HCV-1b infection [Enomoto et al., 1996]. However, it is not known yet whether the region is also a major predictor of the response to IFN therapy in children and adolescents with chronic HCV-1b infection. To clarify whether the number of amino acid changes in $NS5A_{2209–2248}$ could be used as a predictor of response to IFN in young patients with chronic HCV-1b infection, the correlation was examined between mutations in $NS5A_{2209–2248}$ and the outcome following IFN therapy.

MATERIALS AND METHODS

Patients and IFN Regimen

Sixteen children and adolescents with chronic HCV-1b infection (M/F ratio = 11/5; mean age 11.7 ± 4.9 years; range, 5 to 19 years) were enrolled in this study.

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TABLE I. Clinical Characteristics of 16 Pediatric Patients

Gender (M/F)	11/5
Age, yr (mean ± SD)	5–19 (11.7 ± 4.9)
Underlying disease	
Malignant	12
Non-malignant	4
HCV genotype	
1b	12
Co-infection with other HCV genotype	4
Serum HCV RNA levels (copies/mL)	$10^{7.6 \pm 1.4}$
Histologic findings (n = 15)	
G_1 (minimal)	2
G_2 (mild)	7
G_3 (moderate)	6
G_4 (severe)	0
S_0 (no)	2
S_1 (mild)	8
S_2 (moderate)	4
S_3 (severe)	1
S_4 (cirrhosis)	0

G, grade of activity; S, stage of fibrosis.

The clinical characteristics of the 16 patients are summarized in Table I. Four had coinfection with other HCV genotypes. All patients had a history of blood transfusion. Twelve patients had an underlying malignant disease: acute lymphoblastic leukemia (ALL) in seven patients and acute myelogenous leukemia (AML) in five patients. The diagnosis of chronic hepatitis was based on a documented elevation of alanine aminotransferase (ALT) levels for at least 6 months, the presence of anti-HCV antibody, and histologic findings on liver biopsies. All patients were without hepatitis B surface antigen or antibody to hepatitis B core antigen, and with no IgM antibodies against cytomegalovirus and Epstein-Barr virus. Other causes of liver damage, such as α_1-antitrypsin deficiency, hemochromatosis and Wilson's disease also were ruled out. Informed consent was obtained from all of the patients' parents before initiation of IFN therapy. IFN therapy was initiated at least 2 years after completion of treatment for any underlying malignant disease. Three of five patients with AML had undergone bone marrow transplantation. One of seven patients with ALL received a peripheral blood stem cell transplantation. IFN-alpha (Sumitomo Pharmaceuticals Co., Ltd., Osaka, Japan or Otsuka Pharmaceuticals Co., Ltd., Tokyo, Japan) was administered at a dose of 0.1 MU per kilogram (maximum dose:6.0 MU) daily for 2 weeks, and then three times a week for an additional 22 weeks (total dose:8.0 MU/kg). The response to IFN therapy was classified as a "complete response (CR)" if ALT values/HCV RNA returned to normal within 6 months after the cessation of therapy and remained normal for at least 6 months thereafter. A "no response (NR)" was simply defined as any condition other than CR. Anti-HCV antibody was detected using a second generation enzyme immunoassay (Abbott HCV EIA 2.0, Tokyo, Japan). Serum HCV RNA was detected by nested reverse transcription-polymerase chain reaction (RT-PCR) with two pairs of external and internal (nested) primers deduced from the 5′-noncoding region of HCV [Okamoto et al., 1990; Inui et al., 1995]. Serum HCV genotyping was examined before IFN therapy by nested RT-PCR using type-specific primers deduced from the HCV core region [Okamoto et al., 1992]. Serum HCV RNA levels were quantified using a multicyclic RT-PCR method [Ishiyama et al., 1993]. Liver biopsies were obtained from 15 patients before initiation of IFN therapy, and a histologic diagnosis was based on the classification system for chronic hepatitis proposed by Desmet et al. [1994]. Biopsy specimens were examined by an independent pathologist not connected with this study.

RNA Extraction From Serum and RT-PCR

HCV RNA extracted from 50 μL of serum using a guanidinium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987] was reverse-transcribed with 100 pmol of the first antisense primer and 100 units of reverse transcriptase (M-MLV Reverse Transcriptase; Gibco BRL, Gaithersburg, MD) in a final volume of 20 μL. The reaction mixture was incubated at 56°C for 60°C min, heated at 95°C for 5 min, and cooled rapidly. The cDNA was then amplified by PCR with 100 pmol of each oligonucleotide primer described below. All nested PCR reactions were carried out in a DNA thermal cycler (Robocycler 40, Stratagene; La Jolla, CA) with four primers and a Gene Amp kit (Perkin-Elmer Cetus, Norwalk, CT) with 2.5 units of Taq DNA polymerase (Perkin-Elmer Cetus) in a final volume of 100 μL. The first PCR cycle consisted of 34 cycles and the nested PCR cycle consisted of 29 cycles. Each cycle included denaturation at 94°C for 1 min, annealing at 56°C for 1.5 min, and extension at 72°C for 1.5 min.

Oligonucleotide Primers

The region encompassing $NS5A_{2209–2248}$ was amplified by nested PCR using two sets of primers: external sense, 5′-CCGGATGTGGCAGTGCTCA-3′ (nt 6825–6844); external antisense, 5′-CAGCGTTGCCCATATGGGCAC-3′ (nt 7177–7197); internal sense, 5′-TCACCGACCCCTCTCATATTC-3′ (nt 6853–6874); internal antisense, 5′-CGGCGGAGATCCTGCGAAAAC-3′ (nt 7141–7161). The internal antisense primer was labeled with biotin. The primer used for direct sequencing was 5′-CAAGCGTAGGCTGGCCAGGG-3′ (nt 6888–6896) labeled with Cy5 (Pharmacia Biotech, Uppsala, Sweden). Primers were synthesized in an LKB Gene Assembler Plus DNA Synthesizer (Pharmacia Biotech) using the established sequence for HCV-J [Kato et al., 1990]. Labeling was carried out using the same DNA synthesizer.

Direct Sequencing of PCR Products

The amplified PCR products were denatured and the biotin-labeled fragments were recovered using strepta-

TABLE II. Clinical Characteristics of 16 Patients, According to the Response to IFN Therapy

	CR (n = 4)	NR (n = 12)	*P* value
Gender (M/F)	3/1	8/4	.63
Age (yr, mean ± SD)	8.5 ± 3.5	12.8 ± 4.9	.13
Underlying disease			
Malignant	2	10	.14
Non-malignant	2	2	
Serum HCV RNA level (copies/mL)	$10^{7.5 \pm 0.5}$	$10^{7.6 \pm 1.6}$	.85
Grading of liver biopsy (n = 15)			
G_1 (minimal)	0	2	.53
G_2 (mild)	2	5	
G_3 (moderate)	2	4	
G_4 (severe)	0	0	
HCV NS5A sequence			
Wild type	3	5	.47
Intermediate type	0	2	
ND	1	5	

ND, not detected.

TABLE III. Clinical Characteristics of 10 Patients, According to the Types of $NS5A_{2209–2248}$ Sequences Identified

	Wild type (n = 8)	Intermediate type (n = 2)	*P* value
Gender (M/F)	6/2	2/0	.62
Age (yr, mean ± SD)	11.0 ± 5.3	9.0 ± 2.8	.50
Underlying disease			
Malignant	5	2	
Non-malignant	3	0	
Serum HCV RNA levels (copies/mL)	$10^{7.5 \pm 1.1}$	$10^{8.0 \pm 1.4}$	.55
Grading of liver biopsy			
G_1 (minimal)	1	0	.86
G_2 (mild)	4	1	
G_3 (moderate)	3	1	
G_4 (severe)	0	0	
Response to IFN therapy			
CR	3	0	.47
NR	5	2	

vidin-coated magnetic beads (Dynal, Oslo, Norway). The recovered single-stranded DNA was used as a template for sequencing. The nucleotide sequences were determined using a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Life Science Co., Buckinghamshire, England), which is based on the dideoxy chain termination method utilizing Cy5 labeled fluorescent primers. The amino acid sequences were identified using KINOP alignment software (Otsuka-Fujitsu collaborative development; Tokyo, Japan) and compared with published $NS5A_{2209–2248}$ sequences of HCV-J.

Types of Mutations in the NS5A Gene

The mutations in $NS5A_{2209–2248}$ sequences were classified into three groups, according to a previous report by Enomoto et al. [1996]. Wild type was defined as no amino acid changes in this region; intermediate type, 1 to 3 amino acid changes; and mutant type, 4 to 11 amino acid changes.

Statistical Analysis

Results are presented as the mean ± SD. Univariate analysis was undertaken using Fisher's exact test, the Student's *t*-test, and the Mann-Whitney test. A *P* value of .05 or less was considered to represent statistical significance.

RESULTS

Response to IFN Therapy

Four (25%) of the 16 patients responded completely. The clinical characteristics in response to IFN therapy are shown in Table II. Two (50%) of the four patients with CR and 10 (83%) of the 12 patients with NR had an underlying malignant disease. In the CR patients, grading of necroinflammatory activity in liver biopsy specimens was mild in two and moderate in two. In the NR patients, grading of necroinflammatory activity was minimal in two, mild in five, and moderate in four. There was no significant difference in gender, age, underlying disease, and serum HCV RNA levels between the two groups (CR and NR). The results from multicyclic RT-PCR and competitive RT-PCR were similar [Ishiyama et al., 1993]. It was also found the a positive correlation between serum HCV-RNA levels assayed by a branched DNA probe method (Chiron Co., Emeryville, CA) and those determined by multicyclic RT-PCR (data not shown).

Mutations in the NS5A Gene

The $NS5A_{2209–2248}$ sequences were identified in 10 (63%) of the 16 patients. The clinical characteristics in relation to the type of $NS5A_{2209–2248}$ sequences are summarized in Table III. Eight patients (80%) had the wild-type sequence, and two patients (20%) had the intermediate type. Both of these two patients had only one amino acid change. There were no patients with a mutant type with four to 11 amino acid changes. Five (63%) of the eight patients with the wild type had an underlying malignant diseases, whereas both patients with the intermediate type had an underlying malignant disease. Serum HCV RNA levels in relation to the type of $NS5A_{2209–2248}$ sequence showed no significant differences for both groups ($10^{7.5 \pm 1.1}$ vs. $10^{8.0 \pm 1.4}$). In the wild-type group, grading of liver biopsy specimens showed minimal activity in one patient, mild activity in four patients, and moderate activity in three patients. In the patients with the intermediate type, grading showed mild activity in one patient and moderate activity in one patient. Three (38%) of the eight patients with the wild-type sequences achieved CR, compared to none of the two patients with the intermediate-type sequences.

DISCUSSION

Enomoto et al. [1996] reported that all of their adult patients with HCV-1b mutants consisting of four to 11 amino acid changes in the $NS5A_{2209–2248}$ sequence responded completely. Baseline serum HCV RNA levels were also significantly lower in patients with the mutant type (with four to 11 amino acid changes) than in those with the intermediate (with one to three amino acid changes) and wild types.

Their study showed that 54 (64%) of 84 adult patients had at least one mutation in the $NS5A_{2209–2248}$ sequence [Enomoto et al., 1996]. In contrast, in our pediatric patient series, there were no children with the mutant-type $NS5A_{2209–2248}$ sequences, and both patients with the intermediate type of $NS5A_{2209–2248}$ had only one amino acid change. These findings suggest that amino acid sequences in the $NS5A_{2209–2248}$ region are relatively conserved in young patients compared to adults.

In a previous study [Enomoto et al., 1996], all patients with the mutant-type of $NS5A_{2209–2248}$ responded completely, while none with the wild type responded. However, three of the eight children in this study with the wild-type $NS5A_{2209–2248}$ responded completely. These findings suggest that mutations in the NS5A gene are not always associated with sensitivity to IFN therapy in young patients with chronic HCV 1b infection.

Seven of the 10 patients whose amino acid sequences in the $NS5A_{2209–2248}$ region were identified had received intensive immunosuppressive agents for an underlying malignant disease. Although the immune system in children is different from that in adults, it is also possible that immunosuppressive agents, followed by multiple blood transfusions, might affect host immunity against HCV. We suspect that the amino acid sequences in $NS5A_{2209–2248}$ are relatively conserved in these cases.

Four of the 16 patients responded completely. This rate is lower than expected for a 6-month IFN course [Davis et al., 1989; Di Bisceglie et al., 1989; Ruiz-Moreno et al., 1992]. In previous studies [Fujisawa et al., 1995; Komatsu et al., 1996], IFN therapy for children with underlying malignant diseases or infection with genotype 1b was not as effective as in children without those characteristics. The high percentage of patients with underlying malignant diseases or infection with genotype 1b was not as effective as in children without those characteristics. The high percentage of patients with underlying malignant disease and HCV-1b infection might explain this poor response rate. Finally, in our study, no significant correlation was found between amino acid changes in $NS5A_{2209–2248}$ and liver histology.

In conclusion, the data do not provide evidence that mutations in the $NS5A_{2209–2248}$ sequence can be used as a predictor of the response to IFN therapy in children and adolescents with chronic HCV 1b infection. The present study showed no significant correlation between response to IFN and mutations in $NS5A_{2209–2248}$ in young patients. The amino acid sequences in this region of HCV-1b in children, especially those who are immunocompromised, were conserved. However, these results should be confirmed with larger numbers of patients, and further studies are required to determine the relationship between amino acid changes in the NS5A gene and host immunity against HCV-1b.

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